IVD For *in Vitro* Diagnostic Use

CE

Bordetella pertussis/B.parapertussis/ B.bronchiseptica Real-TM

Handbook

Real Time PCR test for detection and differentiation of Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica

REF B84-100FRT

Σ **100**

NAME

Bordetella pertussis/B. parapertussis/B. bronchiseptica Real-TM

INTRODUCTION

Bordetella is a genus of small (0.2 - 0.7 µm), Gram-negativecoccobacilli of the phylum proteobacteria. Three species are human respiratorypathogens (*B. pertussis, B. parapertussis and B. bronchiseptica*).

Bordetella pertussis is an obligate human *pathogen and is the causative agent of whooping cough (pertussis). Bordetella*parapertussis causes a milder form of disease in humans and also causes respiratory infections in sheep. *Bordetellabronchiseptica*has the broadest host range, causing disease in many mammalian species, but kennel cough in dogs and atrophic rhinitis, in which infected piglets develop deformed nasal passages, have the biggest economic impact.Humans,however,arerarelyinfectedwiththisorganism.Wheninfectionsinhumansoccur,theyareoftenacquiredthroughanimalcontactandtypicallyinvolveimmunocompromised patients.Thespectrumofimmunocompromisedpatientswhohavedeveloped*B.bronchiseptica* infectionincludespatientswithmalignanciessuchasHodgkin's disease, chroniclymphocytic leukemia, cardiacand bone marrow transplantation patients,patients with AIDS.

INTENDED USE

Bordetella pertussis/B. parapertussis/B. bronchiseptica Real-TMPCR kit is an in vitro nucleic acid amplification test for detection and differentiation of pathogens that cause whooping cough (*Bordetella pertussis*), parapertussis (*Bordetellaparapertussis*), and Bordetellabronchiseptica infection (*Bordetellabronchiseptica*) in the clinical materials (nasal and oropharyngeal swabs) and culture of microorganisms by using real-time hybridization-fluorescence detection.

PRINCIPLE OF ASSAY

Bordetella determination by the polymerase chain reaction (PCR) with hybridization fluorescent detection includes three stages: DNA extraction from clinical samples, PCR-amplification of pathogen genome specific region and real-time hybridization fluorescent detection. DNA is extracted from samples in presence of Internal Control (IC), which allows to monitor the analysisof each sample.In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of PCRproduct without re-opening the reaction tubes after the PCR run.

During the amplification stage, four simultaneous reactions take place – amplification of the conservative region of ptxA gene that codes pertussis toxin located in Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica genomes; identification of specific regions in genomes of Bordetella pertussis and Bordetella bronchiseptica as well as amplification of nucleic acid sequence of the Internal Control (IC) sample:

Detection channel	FAM/Green	JOE/HEX/Yellow	ROX/Orange	Cy5/Red
Result	IC detection	Detection of pertussis toxin gene, ptxA	Identification of Bordetella pertussis	Identification of Bordetella bronchiseptica

In case of pertussis toxin gene detection (JOE/HEX/Yellow channel), the conclusion about presence of Bordetella spp. (B.pertussis, B.parapertussis or B.bronchiseptica) is made.

If results are concurrently positiveboth in JOE/HEX/Yellow and ROX/Orange channels, the sample is considered positive for Bordetella pertussis.

If results are concurrently positive both inJOE/HEX/Yellow and Cy5/Red channels, the sample is considered positive for Bordetella bronchiseptica.

Presence of Bordetella parapertussis can be concluded in case of pertussis toxin gene detection (JOE/HEX/Yellow channel) in the sample negative for Bordetella pertussis and Bordetella bronchiseptica providing that sufficient Bordetella DNA is available.

MATERIAL PROVIDED

Module No.1: Real Time PCR kit (B84-100FRT)

Bordetella pertussis/B. parapertussis/B. bronchiseptica Real-TM: Real Time amplification

- **PCR-mix-1-FRT Bordetella**, 5 x 0.2 ml
- **PCR-mix-2-FRT**, 0.6 ml
- **TaqF Polymerase,** 0.06 ml
- **Pos Control DNA Bordetella spp.(C+)**, 2 x 0.1 ml
- **Positive Control IC**, 2 x 0.1 ml
- **TE-buffer**, 2 x 0.5 ml
- **Negative Control (C-)***, 1.2 ml
- **Internal Control (IC)****, 2 x 0.6 ml

Contains reagents for 100 reactions (including controls).

* *must be used in the extraction procedure as Negative Control of Extraction.*
** *add 10 µl of Internal Control during the DNA extraction procedure directly to the sample/lysis mixture.*

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit
- Disposable powder-free gloves and laboratory coat
- Automatic adjustable pipettes (from 5 to 20 µl and from 20 to 200 µl)
- Disposable tips with aerosol barriers (100 or 200 µl) in tube racks
- Tube racks
- Vortex mixer/desktop centrifuge
- PCR box
- Real Time PCR instrument
- Disposable polypropylene microtubes for PCR or PCR-plate
- Refrigerator for 2–8 °C, Deep-freezer for ≤ –16 °C

STORAGE INSTRUCTIONS

All components of the**Bordetella pertussis/B. parapertussis/B. bronchiseptica Real-TM** PCR kit (except for PCR-mix-1-FRT, PCR-mix-2-FRT, and TaqF Polymerase) are to be stored at 2–8 ºC when not in use. All components of the **Bordetella pertussis/B. parapertussis/B. bronchiseptica Real-TM** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

PCR-mix-1, PCR-mix-2-FRT and TaqF Polymerase are to be stored at ≤ –16ºC.

PCR-mix-1is to be kept away from light.

STABILITY

Bordetella pertussis/B. parapertussis/B. bronchiseptica Real-TM Test is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than thoseonesstated on the labels may not perform properly and may adversely affect the assay results.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (EN375). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION AND TRANSPORT

Bordetella pertussis/B. parapertussis/B. bronchiseptica Real-TM PCR kitis intended for the analysis ofDNA extracted from:

Posterior nasopharynx swabs. Throat swabs and anterior nasal swabs have unacceptably low rates of DNA recovery and should not be used for pertussis diagnosis. The swab tips may be polyester (such as Dacron®), rayon, or nylon-flocked. Cotton-tipped or calcium alginate swabs are not acceptable as residues present in these materials inhibit PCR assays.

When the material is obtained, place the working part of the swab in a sterile disposable tube with 500 µl of transport medium for storage and transportation of respiratory swabs. Break off the terminal part of the probe to allow tight closing of the tube cup. Close the tube with the solution and the working part of the probe, and mark it.

Oropharyngeal swabs are taken with a probe with a dry viscose swab. Take swabs by rotating the probe over the surface of tonsils, palatine arches, and the posterior wall of the pharynx.

When the material is obtained, place the working part of the swab in a sterile disposable tube with 500 µl of transport medium for storage and transportation of respiratory swabs. Break off the terminal part of the probe to allow tight closing of the tube cap. Close the tube with the solution and the working part of the probe, and mark it.

Storage of clinical specimens is allowed for 3 days at 2–8 °C or during a week at ≤ –16 °C.

Microorganism cultures are resuspended in 1 ml of 0,9 % saline or 0,01 Mphosphate buffer, pH 7,0. The obtained suspension is used for subsequent DNA extraction.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

The following kits are recommended:

- ⇨ **DNA/RNA Prep**(Sacace, REF K-2-9)
- ⇨ **Ribo-Sorb**(Sacace, REF K-2-1)

Please carry out DNA extraction according to the manufacture's instruction.

DNA is extracted from each clinical sample in the presence of **Internal Control** (**10µl** of **IC** is added to each sample).

Transfer **100µl** of **Negative Control** to the tube labeled C–.

PCR PROTOCOL

1. Prepare the required number of tubes for amplification of DNA from clinical and control samples. Thaw the tubes with **PCR-mix-1-FRT*Bordetella*.** Vortex**PCR-mix-1-FRT*Bordetella*,PCR-mix-2-FRT, polymerase (TaqF)** and centrifuge briefly (1-2 sec).
2. Prepare the required number of tubes or strips (including controls) for DNA amplification.
3. For N reactions, add to a new tube:
   - **10*(N+1) µl** of**PCR-mix-1-FRT*Bordetella***,
   - **5.0*(N+1) µl** of **PCR-mix-2-FRT**,
   - **0.5*(N+1) µl** of**TaqF polymerase**.

When calculating the volume of the reaction mixture, take into account controls (negative control of extraction, positive and negative controls of amplification). Refer to Table 1 for calculated reaction volumes:

Table 1. **Reaction mixture preparation scheme**

Reagent volume for one reaction (µl)	**Volume of reagents for indicated number of reactions**		
	10,00	**5,00**	**0,50**
Number of samples (N) to be analyzed*	**PCR-mix-1-FRT*Bordetella*, µl**	**PCR-mix-2-FRT, µl**	**TaqF Polymerase, µl**
2	60	30	3,0
4	80	40	4,0
6	100	50	5,0
8	120	60	6,0
10	140	70	7,0
12	160	80	8,0
14	180	90	9,0
16	200	100	10,0

**Number of samples including DNA extraction and amplification controls (N+4).*

4. Mix the tube with the prepared mixture and vortex it briefly.
5. Transfer **15 µl** of the prepared mixture to prepared tubes.
6. Add **10 µl** of **DNA** obtained after nucleic acid extraction from clinical or control samples.
7. Carry out the control amplification reactions**:**

NCA - Add **10 µl** of **TE-buffer** to the tube labeled NCA (Negative control of amplification).

C+ - Add **10 µl** of **Positive Control DNA *Bordetella* spp.** to the tube labeled C+(Positive control of amplification).

IC+ - Add **10 µl** of **Positive Control IC**to the tube labeled CS+ (Positive control of IC amplification).

C- - Add **10 µl** of a sample extracted from the **Negative Control** to the tube labeled C- (Negative Control of Extraction).

It is recommended to sediment drops from walls of tubes by short vortex (1–3 s) before inserting them in the thermalcycler.

Amplification

1. Create a temperature profile on your instrument as follows:

	Rotor-type instruments[1]			Plate-type instruments[2]		
Step	**Temperature, °C**	**Time**	**Cycles**	**Temperature, °C**	**Time**	**Cycles**
1	95	15 min	1	95	15 min	1
2	95	10 s	10	95	10 s	10
	60	20 s		60	25 s	
	72	10 s		72	25 s	
3	95	10 s	35	95	10 s	35
	60	20 s Fluorescence detection		60	30s Fluorescence detection	
	72	10 s		72	25 s	

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*
[2] *For example, iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7500 (Applied), SmartCycler® (Cepheid), SaCycler-96™(Sacace)*

Fluorescent signal is detected in FAM/Green, JOE/Yellow/HEX, ROX/Orange, and Cy5/Red channels.

2. Place PCR tubes into the PCR instrument.
3. Run amplification and signal detection program.
4. After measurement, start data analysis and interpretation of results.

INSTRUMENT SETTINGS

Rotor-type instruments(RotorGene 6000/Q)

Channel	Threshold	Calibrate / Gain Optimisation…	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*0.1*	from 5Fl to 10Fl	*0 %*	*Off*
JOE/Yellow	*0.1*	from 5Fl to 10Fl	*5 %*	*Off*
Rox/Orange	*0.1*	from 5Fl to 10Fl	*5 %*	*Off*
Cy5/Red	*0.1*	from 5Fl to 10Fl	*5 %*	*Off*

Plate- or modular type instruments (SaCycler, iQ5, Mx300P, ABI 7500, SmartCycler)

For result analysis, set the threshold line at a level corresponding to 10–20% of the maximum fluorescence signal obtained for Pos C+ sample during the last amplification cycle.

DATA ANALYSIS

The results are analyzed by the software oftheused real-time PCR Instrument. Interpretation of the results is performed by the crossing (or not) of the fluorescence curve with the adjusted threshold line for each single fluorescent channel employed. It defines presence (or absence) of threshold cycle (Ct) for a certain DNA sample in the according graph of the resultant table.

Principle of interpretation of results:

PCR curves of accumulatedfluorescent signal are analyzed in four channels:

Detection channel	FAM/Green	JOE/HEX/Yellow	ROX/Orange	Cy5/Red
Result	IC detection	Detection of pertussis toxin gene, ptxA	Identification of Bordetella pertussis	Identification of Bordetella bronchiseptica

– in the **JOE/HEX/Yellow** channel, signal indicating amplification of pertussis toxin gene fragment present in genomes of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* is registered;
– in the **ROX/Orange**channel, signal indicating amplification of genome's specific region of *Bordetella pertussis* is registered;
– in the **Cy5/Red** channel, signal indicating amplification of genome's specific region of *Bordetella bronchiseptica* is registered;
– in the **FAM/Green** channel, signal indicating amplification of Internal Controlis registered.

The result of amplification is considered to be **positive** if the real-time PCR fluorescent curve is typically S-shaped, crosses threshold line in the area of reliable increment of fluorescence, and Ct value in the results grid for a particular channel is less than the indicated boundary value, **negative** – in case of absence of typically S-shaped curve not crossing the threshold line (does not have Ct), or Ct exceeds the indicated boundary value.

The results of analysis are considered reliable only if the results obtained for positive and negative controls of amplification and negative control of extraction are correct (see Table 2).

Table 2. **Results for controls**

Control	Stage for control	Value of threshold cycle, *Ct*			
		FAM/Green	JOE/HEX/ Yellow	ROX/Orange	Cy5/Red
		IC	Pertussis toxin gene, *ptxA*	*Bordetella pertussis*	*Bordetella bronchiseptica*
NCE	DNA extraction	**<30**	Neg	Neg	Neg
NCA	Amplification	Neg	Neg	Neg	Neg
IC+	Amplification	**<27**	Neg	Neg	Neg
C+	Amplification	Neg	**<25**	**<27**	**<26**

Principle of interpretation of results:

Interpretation of results of PCR analysis for detection and differentiation of pathogens that cause whooping cough (*Bordetella pertussis*), parapertussis(*Bordetellaparapertussis*), and Bordetellabronchisepticainfection (*Bordetellabronchiseptica*) is based on amplification results in accordance with Table 3.

Table 3. **Interpretation of results for PCR-analysis**

Variants	FAM/Green	JOE/HEX/ Yellow	ROX/ Orange	Cy5/Red	Interpretation
	Value of threshold cycle, *Ct*				
1	absentor >29	absentor >25	absent	absent	**Invalid**
2	<29	absent	absent	absent	*B.pertussis* *B.parapertussis* *B.bronchiseptica* **NOT detected**
3	≤35 or absent	≤35	≤35	absent	*B.pertussis* DNA **detected**
4	≤35 or absent	≤35	absent	≤35	*B.bronchiseptica* DNA **detected**
5	≤35 or absent	<25	absent	absent	*B.parapertussis* DNA **detected**
6	<29	>25	absent	absent	*Bordetella* spp. DNA **detected**: *B.pertussis,* or *B.parapertussis,* or *B.bronchiseptica.* For differentiation, repeat sampling and extraction.
7	<29	absent	≤35	absent	If the result is repeated in PCR, interpret as **equivocal**
8	<29	absent	absent	≤35	If the result is repeated in PCR, interpret as **equivocal**

- *Bordetella pertussis*, *Bordetellaparapertussis* and *Bordetellabronchiseptica* is **not detected** in a sampleif the *Ct* value of a sample specified in the table for the **HEX,ROX** and**Cy5** channels has not been detected (absent), i.e. the PCR florescent curve does not cross the threshold line. Herewith, *Ct* value for internal control (IC) (**FAM**) does not exceed 29.
- *Bordetellapertussis* DNA is **detected** in a sample if the *Ct* value of a sample specified in the table for the**HEX** and **ROX** channelsis less or equal to 35, and the PCR fluorescent curve should have typical exponential increase of fluorescent signal. In such samples, *Ct* for internal control (IC) (**FAM**) can have any value or be absent in case of high pathogen load in a sample.

– *B. bronchiseptica* DNA is **detected** in a sample if the *Ct* value of a sample specified in the table for the **HEX** and **Cy5**channels is less or equal to 35, and the PCR fluorescent curve should have typical exponential increase of fluorescent signal. In such samples, *Ct* for internal control (IC) (**FAM**) can have any value or be absent in case of high pathogen load in a sample.

– *B. parapertussis* DNA is **detected** in a sample if the *Ct* value of a sample specified in the table for the **HEX** channel does not exceed 25, and *Ct* values for the **ROX** and **Cy5** channels are absent. In such samples, *Ct* for internal control (IC) (**FAM**) can have any value or be absent in case of high pathogen load in a sample.

– *Bordetella* spp. (*B. pertussis,* or *B. parapertussis,* or *B. bronchiseptica*) DNA is **detected** if *Ct* value of a sample specified in the table for the **JOE/Yellow** channel exceed 25, *Ct* values for the **ROX** and **Cy5** channels are absent, *Ct* for internal control (IC) (**FAM**) does not exceed 29. That can take place if the quantity of extracted DNA is insufficient. For species differentiation, repeat sampling and nucleic acid extraction steps.

– Results should be interpreted as **equivocal** if a repeated PCR analysis demonstrates absent *Ct* value in the **HEX** channel, while *Ct* values in the **ROX** (or **Cy5**) and **FAM** channels do not exceed 29. Repeat of sampling is advised.

– Results should be interpreted as **invalid** if the *Ct* values of a sample specified in the table for the **ROX** and **Cy5** channels have not been detected (absent), for the **HEX** channel – is absent or exceed 25, and for internal control (IC) (**FAM**) - is either absent or exceed 29. In this case, repeat nucleic acid extraction step for the required clinical sample.

Results of analysis are not taken into account in the following cases:

– If Ct value of positive control DNA *Bordetella* spp. (C+) in the appropriate channel is absent or exceeds boundary value (see Table 3), repeat amplification for all negative clinical samples.

– The samples with negative result in all channels should be analyzed once more starting from the nucleic acid extraction stage. If negative result is obtained in the second run, repeat collection of the clinical material. Negative result is normally detected only for the Negative Control of amplification (NCA).

– If the Ct value for the Positive Control of amplification (C+) is absent or exceeds the boundary *Ct* value in the appropriate channel, repeat amplification for all negative clinical samples.

– If the *Ct* value is present for the Negative Control of extraction (C–) and/or Negative Control of amplification (NCA) in the channel for detection of any gene target (**HEX,ROX** and**Cy5**), repeat the analysis for all samples in which the specific gene was detected starting from the nucleic acid extraction stage to rule out possible contamination.

SPECIFICATIONS

Sensitivity

The analytical sensitivity of **Bordetella multiplex Real-TM**PCR kit for the stated causative agents is no less than $5x10^2$copies per 1 ml of sample.

Specificity

Bordetella multiplex Real-TMPCR kit makes it possible to detect DNA specific regions of the statedcausative agents. The specificity of this kit was confirmed by investigation of the following reference strains: *Streptococcus*spp*., Moraxella catarrhalis, Staphilococcus aureus, Staphilococcus saprophiticus, Haemophilus influenzae, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Mycobacteria tuberculosis* 27294 105*, Neisseria flava, Neisseria sicca, Neisseria mucosa, E. coli ATCC, NCTC,* 01577 27u7*, Enterococcus faecalis, Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila, Shigella flexneri, Shigella sonnei, Salmonella Enteritidis, Yersinia enterocollitica* – as well as human genomic DNA.

TROUBLESHOOTING

Results of analysis are not taken into account in the following cases:

1. If Ct value of positive control DNA *Bordetella* spp. (C+) in the appropriate channel is absent or exceeds boundary value, amplification stage is to be repeated for all clinical samples interpreted as negative.
2. If Ct value of negative control of extraction (C-) in the **JOE/HEX/Yellow,ROX/Orange** and**Cy5/Red** channels and/or negative control of amplification (NCA) in any fluorescent channel is detected, test analysis must be repeated from the extraction stage and measures to detect and eliminate the source of possible contamination must be taken.
3. If a positive result (the fluorescence curve crosses the threshold line) is detected for a sample that has a fluorescence curve without the typical exponential growth phase (the curve is linear), this may suggest incorrect setting of the threshold line or incorrect calculation of baseline parameters. Such a result should not be considered as positive.

If you have any further questions or if you encounter problems, please contact our Authorized representative in the European Community.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
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